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Methods designed for the identification and characterization of *in vitro* and *in vivo* chromatin assembly mutants in *Saccharomyces cerevisiae*

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ABSTRACT

Assembly of DNA into chromatin allows for the formation of a barrier that protects naked DNA from protein and chemical agents geared to degrade or metabolize DNA. Chromatin assembly occurs whenever a length of DNA becomes exposed to the cellular elements, whether during DNA synthesis or repair. This report describes tools to study chromatin assembly in the model system *Saccharomyces cerevisiae*. Modifications to an *in vitro* chromatin assembly assay are described that allowed a brute force screen of temperature sensitive (*ts*) yeast strains in order to identify chromatin assembly defective extracts. This screen yielded mutations in genes encoding two ubiquitin protein ligases (E3s): *RSP5*, and a subunit of the Anaphase Promoting Complex (APC), *APC5*. Additional modifications are described that allow for a rapid analysis and an *in vivo* characterization of yeast chromatin assembly mutants, as well as any other mutant of interest. Our analysis suggests that the *in vitro* and *in vivo* chromatin assembly assays are responsive to different cellular signals, including cell cycle cues that involve different molecular networks.

INTRODUCTION

DNA is the most evolutionarily conserved molecule. Most, but not all systems that encode proteins, from viruses and bacteria to higher eukaryotes, do so by interpreting the information stored within DNA. It is therefore no surprise to learn that virtually all proteins that interact with DNA, such as histones for example, are also highly conserved evolutionarily. Recent studies have shown that the interaction between histones and DNA, to form chromatin, are extremely dynamic and serve as a platform for post-translational modifying activities, such as acetylation, phosphorylation, methylation and ubiquitination (1, 2). In the absence of proper assembly, regulation and maintenance of chromatin, the cell will most likely die due to misregulated transcriptional control or defects in chromosome folding and packaging.

Chromatin is made up of segments of approximately 146 basepairs of DNA wrapped around a complex of eight proteins

made up of two copies of the four histones, H2A, H2B, H3 and H4 (3, 4). This complex is also known as the nucleosome. Chromatin assembly is initiated by a tetramer of two H3 molecules and two H4 molecules, which interact with naked DNA with the aid of chromatin assembly factors. Two H2A/H2B dimers are then delivered to the H3/H4/DNA complex by other chromatin assembly factors to complete the nucleosome (5). *In vitro* biochemical studies have demonstrated that histones incorporated into chromatin are acetylated and that once assembled, the chromatin is matured by the removal of the acetyl groups (6, 7). Deacetylation of newly assembled histones is generally believed to play an important role in the establishment of heterochromatin (reviewed in 4). Subsequent action by a host of histone modifying activities direct transcription, replication, DNA repair, chromosome condensation and decondensation, and ultimately, cell cycle progression (8-10).

To understand chromatin assembly at a molecular level an *in vitro* chromatin assembly assay was developed that relied on a simple plasmid supercoiling assay and a yeast whole-cell extract (11). We showed that this assay depended on ATP, acetylatable histone tails and was cell cycle regulated (11-15). The use of this system lead to a genetic screen aimed at identifying factors involved in chromatin assembly. This resulted in the discovery of the ubiquitin system as a major player in regulating chromatin assembly (15).

The ubiquitin targeting system relies on three classes of proteins to select and ubiquitinate proteins: a ubiquitin-activating protein (E1), one of a family of ubiquitin-conjugating proteins (E2), and one of a structurally and functionally distinct family of ubiquitin-protein ligases (E3; 16). Our studies revealed that the E3 enzymes, Rsp5p and the Anaphase Promoting Complex (APC), are required for an *in vitro* chromatin assembly activity that is most active during mitosis (14, 15). The discovery of both Rsp5p and the APC in our screen presented an interesting challenge. For example, Rsp5p, a single polypeptide activity, is localized to the plasma membrane and adjacent to vacuoles (17, 18), whereas the APC, an evolutionarily conserved complex of at least 13 proteins, functions within the nucleus (19, 20). Furthermore, Rsp5p is involved in mitochondrial biogenesis (21, 22), plasma membrane protein turnover, endocytosis, transcription and recombination (reviewed in 23), while the main function of the APC is believed to be the targeting of proteins that inhibit mitotic progression and exit for degradation (24, 25). It is possible that the identification of Rsp5p and the APC in our screen reflects the existence of a signaling cascade controlling chromatin assembly that connects extracellular signals with chromatin metabolism.

In this report, we describe in detail the methods we utilized to study chromatin assembly, both *in vitro* and *in vivo*. These methods allowed us to carry out a brute force genetic screen that resulted in the isolation of chromatin assembly mutants in yeast. Furthermore, we describe additional modifications to these assays that can be used to screen for additional chromatin assembly mutants or characterize existing mutants that may play a role in chromatin metabolism.

MATERIALS AND METHODS

Yeast strains and culture media

The yeast strains used in this study are shown in Table 1. The H1G4 strain (YTH335) contains the mutations *rmc1/apc5*^{CA}, *rmc2* and *rmc3/rsp5*$^{P-S}$ (15). Cells were cultured in the media indicated: YPD (1% yeast extract, 2% peptone and 2% glucose [Difco]) and YPGal (1% yeast extract, 2% peptone and 2% galactose). Hydroxyurea (Sigma) was added to cells growing in YPD to a final concentration of 0.3 M. Methods for preparing yeast cells for FACS scan analysis have been described previously (26).

Table 1: Yeast strains used in this study

Strain	Genotype	Source
RMY102	*MAT***a** *ade2-101 his3-Δ200 lys2-801 trp1Δ901 ura3-52 hht1 hhf1::LEU2 hht2 hhf2::HIS3* + pRM102 (*CEN/URA3 GAL10*$_{prom}$*-HHT2 Gal1*$_{prom}$*-HHF2*)	M. Grunstein (12, 31)
S288c derivatives		
YTH3	*MATα ade2 leu2-3,112 lys2Δ201 ura3-52*	(15)
YTH6	*MAT***a** *ade2 his3Δ200 lys2Δ201 ura3-52*	(15)
YTH1235	*MAT***a** *ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52*	YTH3 x YTH6
YTH1155	*MATα ade2 his3Δ200 leu2-3,112 lys2Δ201*(?) *ura3-52 apc5*^{CA}-PA*::His5*$^{+}$	(15)
A354a derivatives		
YTH83	*MAT***a** *ade1 ade2 gal1 his7 lys2 tyr1 ura1*	M. Winey
H1F1-H1G6	isogenic to A364a	Hartwell *ts* library (27)
YTH335 (H1G4)	*MAT***a** *ade1 ade2 gal1 his7 lys2 tyr1 ura1 rmc1-1 rmc2 rmc3-1*	(15)

Whole-cell extract preparation

"Noodle" preparation

For the screen presented in this report, one hundred strains were chosen at random for extract preparation from the Hartwell collection of temperature sensitive (*ts*) strains (27). Extract preparations involve three main steps: i) generation of frozen "noodles," ii) grinding of the "noodles," and iii) ultracentrifugation of the ground powder. To prepare yeast "noodles," strains of interest are picked from glycerol stocks stored at -80°C and grown on YPD plates. Fresh colonies from

these plates are then grown in 10 ml YPD and used to inoculate 500 ml YPD cultures. The cultures, usually grown in batches of six (the ultracentrifuge rotor holds 6 samples), are shaken at room temperature until an OD_{600} of 3.0 is reached, then shifted to 37°C for two hours. The cells are then harvested by centrifugation at 4,000 rpm for 5 minutes. All supernatant is removed and the mass of the cell pellet is determined. The cells are then washed, in succession, with i) 10 ml H_2O, ii) 10 ml YEB (yeast extract buffer; 1 mM EDTA, 5 mM EGTA and 100 mM HEPES pH 7.9), and iii) 1.3 x volume/cell mass YEB plus a protease inhibitor cocktail (0.2 mM PMSF, 10 mM Benzamidine-HCl, 25 µg/ml TPCK, 5 µg/ml leupeptin, 3.5 µg/ml pepstatin and 10 µg/ml aprotinin) and 2.5 mM DTT. The protease inhibitors are purchased from Sigma. All buffers are kept at 4°C and the protease inhibitor cocktail is diluted fresh from frozen stocks (200 mM PMSF in isopropanol [kept at room temperature], 1 M Benzamidine in H_2O, 3 mg/ml TPCK in ethanol, 1 mg/ml leupeptin in H_2O, 1 mg/ml pepstatin in methanol, 2 mg/ml aprotinin in 0.1% NaCl and 1M DTT in 10 mM NaAc pH 6). The final yeast pellets are then loaded into 10 ml syringes that are pre-cooled in a 4°C cold room. The yeast filled syringes are then extruded into 50 ml Falcon tubes filled with liquid nitrogen. The yeast "noodles" can then be stored at -80°C indefinitely.

Whole-cell yeast extract preparation from a library of ts yeast cells

We generally prepare extracts from the yeast "noodles" in batches of six. The entire process is best carried out in a cold room. To begin, 2.5 g of frozen "noodles" (fragmented by shaking) are added to 250 ml beakers that are pre-cooled on dry ice for weighing purposes. The "noodle" fragments are then added to coffee mills (standard coffee mills purchased at any home hardware store) that are pre-cooled with ground dry ice pellets. Dry ice is added to the coffee mills so that the ground powder just covers the mill blades. The yeast "noodle"/dry ice powder mixture is ground for 5 minutes. When the grind is finished the mills are quickly turned upside down to gather the ground yeast powder in the cap. This also avoids thawing the yeast in the rapidly warming coffee mills. The yeast powder is then scooped into the 250 ml beakers (which should be at 4°C), the remaining dry ice powder is dissipated and 1.3 x volume/mass YEB plus protease inhibitor cocktail and DTT is added. A suspension of YEB and yeast powder is then made by pipetting. The remaining yeast powder on the beaker walls can be washed in this manner. The mixture is added to cooled ultracentrifugation tubes (Beckman) and the weight of each tube is determined to ensure opposed tubes are perfectly balanced. Unbalanced tubes can be equilibrated by the additional of mineral oil to the lighter tube. The balanced tubes are loaded into a cooled ultracentrifuge rotor (SW55Ti) and spun for 2 hours at 27,000 rpm (100,000 x g). In our hands, extracts prepared from samples where a low speed spin was used rather than a high speed spin were unsuccessful.

During the ultracentrifugation, a 50X volume of YDB/volume of total extract is prepared (yeast dialysis buffer; 20 mM HEPES pH 7.9, 0.05 mM EDTA, 5 mM EGTA, 20% glycerol, 2.5 mM DTT, 0.2 mM PMSF and 0.5 µg/ml leupeptin) and stored at 4°C with a gently spinning stir bar. Dialysis tubing (MWCO: 6-8,000; Spectrum Laboratories, Inc.) is prepared by cutting strips of approximately 6 inches for each sample. One end of the tubing is folded over and clamped, and then the tubing is added to the YDB until the ultracentrifuge spin is complete. At the end of the spin the whole-cell protein extract is removed by tube puncture. The soluble protein sediments between a pellet composed of cellular debris layered with genomic DNA and a top layer consisting of lipids. A needle (22G x 1"; Terumo Medical Corp., Elkton, MD) attached to a 5 ml syringe is typically used to carefully puncture the tube just above the cell debris pellet. The soluble protein is removed and added to the cooled dialysis tubing. The free end of the dialysis tubing is clamped and the extract dialyzed against YDB at 4°C for at least 3 hours. At the end of the dialysis, the extract is added to microfuge tubes in 50-100 µl aliquots on ice. The microtubes are then flash frozen in liquid nitrogen and stored at -80°C indefinitely.

Genetic screen for chromatin assembly mutants

Chromatin assembly reactions were performed on extracts prepared from temperature sensitive (*ts*) strains according to previously published protocols (11, 15). Here, we describe modified protocols that allowed the *in vitro* assay to be combined with a brute force genetic screen.

^{32}P-labeled plasmid DNA preparation

In a typical chromatin assembly reaction, 20-100 µg of whole-cell extract protein is incubated with 20 ng of uniquely labeled circular, relaxed pBluescript (pBS) plasmid. The labeled probe is prepared by digesting 25 µg of pBS with *Hind*III in a 50 µl reaction. The terminal phosphates from the *Hind*III digested pBS are removed by calf intestinal phosphatase (CIP) treatment; 50 µl *Hind*III/pBS, 20 µl 10X CIP buffer, 2.5 µl CIP (New England BioLabs) and 128 µl H_2O are incubated at 37°C for 1 hour. The DNA is then precipitated by the addition of 20 µl 3 M NaAc, pH 4.8, and 500 µl 95% ethanol. The mixture is incubated at -80°C for 15 minutes, centrifuged for 10 minutes, and the dried pellet resuspended in 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) at a concentration of 0.25 µg/µl. The *Hind*III digested pBS is end-labeled by incubating 0.5 ug (in 2 µl) of linear plasmid with 7.5 µl of ^{32}P-γ-ATP (10 µCi/µl), 1.2 µl of 10X T4 polynucleotide kinase buffer and 1 µl of T4 polynucleotide kinase (New England BioLabs). The reaction is incubated at 37°C for one hour and then added to a 500 µl ligation reaction containing the 12 µl kinase reaction, 50 µl 10X T4 ligase buffer and 3 µl T4 ligase (New England BioLabs). The ligation reaction is continued overnight at 16°C. The next day, ligated, and uniquely labeled pBS is purified by chloroform/phenol and chloroform extraction. The top layer of each extraction (500 µl) is kept and 67 µl of 5 M NH_2SO_4 is added following the extractions. The mixture is split into two tubes and the probe is precipitated by the addition of 95% ethanol (800 µl) to each tube. The

precipitated "hot" DNA is pelleted and resuspended in a total of 25 µl H_2O (20 ng/µl). A 50 µl "hot/cold" mix is then prepared by mixing 5 µl of ^{32}P labeled "hot" pBS with 400 ng (100 ng/µl) of a "cold", relaxed, circular DNA preparation in H_2O. "Cold" relaxed DNA is prepared by treating 50 µg of supercoiled pBS with 50 units of topoisomerase I in a 100 µl reaction, which is incubated at 37°C for 1.5 hours. The relaxed "cold" DNA is precipitated as described above and resuspended in a total of 500 µl H_2O. All DNA preparations are stored at -20°C.

In vitro chromatin assembly reactions and analysis

A typical chromatin assembly reaction is composed of 2 µl "hot/cold" DNA, 1 µl ATP (from a 60 mM stock in H_2O pH 7.5; Sigma), 1 µl creatine kinase (from a 2 µg/ml stock in 1 mM Tris-HCl pH 7.5; Sigma), 1 µl phosphocreatine (from a 400 mM stock in 1 mM Tris-HCl pH 7.5; Sigma) and 2 µl CAB (10 X chromatin assembly buffer; 75 mM $MgCl_2$, 10 mM DTT and 0.5 mM EDTA) for a total of 7 µl, which is then brought up to 20 µl as follows. For each whole-cell extract, the volume required for 20-100 µg protein is calculated, with the balance made up with YDB. The final volume of 20 µl therefore consists of a 7 µl cocktail mix plus a 13 µl mix of YDB and protein extract. The extract is added after the YDB. The reaction tubes are kept on ice during this entire process. Once the extract is added, the reactions are incubated at 30°C for 30-60 minutes. The reactions are stopped by the addition of 200 µl of stop buffer (0.3 M NaAc pH 6.0, 0.5% SDS and 10 mM EDTA). The assembled template is extracted by the addition of 200 µl phenol/chloroform, and precipitated by the addition of 500 µl 95% ice-cold ethanol. The precipitated DNA pellet is dried and resuspended in 10 µl of 1X DNA loading dye. The samples are then separated through a large 0.8% agarose gel (13 x 25 cm; Owl) run overnight at 70 volts in 1X TAE (50X stock: 242 g/L Tris base, 57.1 ml/L glacial acetic acid, 100 ml/L 0.5 M EDTA pH 8.0). The next morning the gel is stained in ethidium bromide, photographed and dried on a standard gel drier (Labconco; VWR). The dried gel is exposed to X-ray film (Kodak) overnight and developed the next day. Extracts found to be defective for *in vitro* chromatin assembly are repeated at least 3 times, from both heat shocked and non-heat shocked *ts* mutants. Extracts found to be defective in all three trials are deemed to be true chromatin assembly mutants. Assembly efficiency is found to be variable between independent extracts and between individual reactions with the same extract. Therefore, at least three independent reactions are performed with each extract. A sample of chromatin assembly run on 18 extracts prepared from the Hartwell *ts* strains is shown in Figure 1. Characterization of the H1G4 (YTH335) strain has been reported (15).

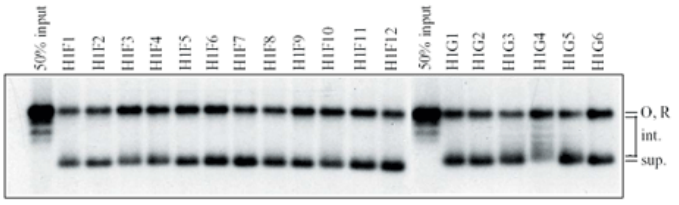

Fig. 1: A brute force genetic screen for *in vitro* chromatin assembly mutants in yeast. Whole-cell extracts were prepared from yeast strains randomly selected from a library of temperature sensitive (*ts*) mutants (27). 100 µg of extract protein was incubated with uniquely labeled circular relaxed plasmid DNA in the presence of an ATP regeneration system. Following the reaction, the intermediate DNA topoisomers (int.) were resolved, together with the open, relaxed (O, R) and supercoiled (sup.) species, through a 0.8% agarose gel. An extract is deemed to be defective if an accumulation of intermediate topoisomers is observed. One extract, H1G4, was found to be unable to fully supercoil the input plasmid (compare the accumulation of intermediate topoisomers to completely supercoiled bands).

Non-radioactive *in vitro* chromatin assembly

The whole-cell extract preparation described above also supports *in vitro* chromatin assembly in the presence of non-radioactive probe DNA. Typically, 100-200 ng circular, relaxed pBS (relaxed by topoisomerase treatment described above) is incubated with 150-200 µg protein extract (see above for description of extract preparation). The reaction is generally performed in a final volume of 40 µl to accommodate the protein extract. ATP, phosphocreatine and creatine kinase is added from a 20X stock, while CAB buffer is added from a 10X stock. The incubations are performed at 30°C for 1 to 2 hours. The reaction is stopped and the reaction products are isolated and analyzed as above, except the topoisomers are visualized by ethidium bromide staining.

If removal of protein and RNA from the reaction is desired prior to gel analysis, RNase A and protease K are added as follows. The chromatin assembly reactions are stopped by the addition of 10 µl of 2.5% Sarkosyl, 100 mM EDTA. Next, 1.0 µl of RNase A, from a 10 mg/ml stock (200 µg/ml final) is added, followed by a 15 minute incubation at 37°C. Following this, 5.0 µl of protease K from a 10 mg/ml stock (1 mg/ml final) is added, with a continued incubation at 37°C for 30 minutes. As above, 200 µl of stop buffer is added and the DNA is extracted and precipitated.

In vivo chromatin assembly

In vivo chromatin assembly assesses the degree of histone deposition onto the endogenous yeast 2µ plasmid (28, 29). 2µ plasmid DNA is isolated from 10 ml yeast cultures grown in the appropriate media, which is typically YPD. The 2µ plasmid is isolated by performing a standard "smash and grab" protocol. Briefly, cells from the 10 ml cultures are pelleted and resuspended in 200 µl SCE buffer (1 M Sorbitol, 100 mM $NaPO_4$ pH 7.0 and 60 mM EDTA) containing lyticase (10 mg lyticase/3 ml SCE; Sigma) to degrade the yeast cell wall. Zymolyase can be substituted for lyticase at equal quantities with similar results. The cells are then incubated on the bench top and checked for lysis every 15 minutes by mixing 5 µl of H_2O with 2 µl of cells on a microscope slide. Typically, lysis requires 30-40 minutes. When the cells are lysed, 400 µl of lysis buffer (80 µl 10 N NaOH, 400 µl 10% SDS and H_2O up to 4 ml) is added and incubated on ice for 5 minutes. Next, 300 µl of 3M NaAc pH 4.8 is added and the tubes are incubated on ice for 5 minutes. The mixture is then centrifuged at 14,000 rpm for 5 minutes, with 600 µl of isopropanol added to the supernatant. A 10-minute incubation on ice follows. The tubes are then centrifuged for 10 minutes at

14,000 rpm and the pellet is washed with 500 µl of 70% ethanol. Finally, the dried pellet is resuspended in 50 µl H_2O.

The 2µ plasmid topoisomers are separated through a large 0.8% agarose gel (13 x 25 cm; Owl) run at 70 V overnight in 1X TPE buffer (10X TPE: 108 g/L Tris base, 15.5 ml/L 85% phosphoric acid, 40 ml/L 0.5 M EDTA) in the dark. Chloroquine (20 mg/ml stock, stored in the dark, kept less than 4 weeks at 4°C; Sigma) is added to the running buffer and gel at concentrations ranging from 1.75-2.5 µg/ml. The appropriate concentration of chloroquine is determined qualitatively from observing the degree of negative supercoils resulting from a given concentration of chloroquine and adjusted in subsequent gels. Chloroquine loses its effectiveness over time. Therefore, fresh batches of stock chloroquine must be maintained. The 2µ topoisomers are observed by hybridizing ^{32}P-labeled *REP1* DNA, which is encoded within the 2µ plasmid, to 2µ sequences transferred to nitrocellulose according to standard protocols (30). *REP1* DNA is generated by PCR using the following primers: *REP1*-N: GCTAGAATTCGAGCTCATGAATGGCGAGACTGC; *REP1*-C: GCTAGGTACCTCACCCATCCACCTTTCGCTC. The underlined sequence in the *REP1*-N primer defines *Eco*RI and *Sac*I restriction sites, whereas the underlined sequence in the *REP1*-C primer defines a *Kpn*I restriction site. These sites were included in the primers for cloning purposes. Loading of 2µ DNA is adjusted in subsequent gels according to visualization of 2µ DNA.

RESULTS AND DISCUSSION

We have developed *in vitro* and *in vivo* methods for the study of chromatin assembly in yeast. As the basics of the *in vitro* and *in vivo* methods have been published previously (11, 28), the aim of this report is to describe modifications made to the methods that i) facilitate large-scale library screens, and ii) allow a much more rapid analysis. We will also describe mechanistic differences between the *in vitro* and *in vivo* methods that suggest alternative molecular control.

Large-scale library screen for yeast chromatin assembly mutants using extracts prepared from kitchen coffee mills

Protein extracts are prepared from yeast according to many different protocols. The majority of these methods are rapid and allow analysis of cellular proteins within hours by Western analysis (30). In our hands, methods that utilize glass beads or enzymatic breakage of the yeast cell wall to allow extraction of soluble proteins do not support *in vitro* chromatin assembly. The only method that we have found to extract whole cell proteins from yeast that will support *in vitro* chromatin assembly is by breakage of the cell wall in a kitchen coffee mill in the presence of dry ice. The key aspects of this protocol that must be strictly adhered to include: i) maintaining the yeast at or below 4°C at all times; ii) starting with at least 2 grams of pelleted cells; and iii) including a protease inhibitor cocktail in all washing and extraction buffers.

Routinely, six yeast strains can be ground in coffee mills at one time. The limiting factor to the number of strains processed is the number of tubes that can be held in the ultracentrifuge rotor. If two ultracentrifuges are available, preparation of 12 extracts from 500 ml cultures is not overly cumbersome. However, if screening a library (usually a minimum of 100 extracts), daily preparation of 12 extracts may become onerous. As noted in the Materials and Methods, the frozen cell "noodles" can be made ahead of time and stored at -80° for an indefinite amount of time. If the approach is taken that all "noodles" are prepared ahead of time and frozen, six extracts can be prepared per day, totaling 30 per week. It is therefore possible to prepare 100 extracts per month (not including the time required for "noodle" preparation).

During the extract preparation, there are blocks of time required for centrifugation and dialysis. On a typical extraction day (assuming six extracts), approximately 60 to 90 minutes is required to prepare and grind the yeast "noodles," thaw the broken yeast powder and to beginning ultracentrifugation. The ultracentrifuge spin is for 2 hours at 27,000 rpm (100,000 x g). Following the centrifuge spin, the extracts are dialyzed against a weak salt buffer. Setting up the dialysis requires approximately 30 minutes and the dialysis is for 3 to 4 hours. Dispensing the resultant extract and flash freezing in liquid nitrogen will take approximately 30 minutes. Therefore, allow approximately 8 hours to complete the preparation of six whole-cell extracts. If you desire to test the extracts for assembly activity as you go, there is plenty of time during the centrifuge spin and dialysis to conduct the chromatin assembly reactions. Figure 1 illustrates a typical assembly reaction using (γ-^{32}P) ATP-labeled plasmid DNA from 18 extracts in which one extract was assembly defective. If "noodles" are prepared when needed, then 18 extracts per week (3 days of six extracts each) is achievable. The total time required for the experiment shown in Fig. 1 from seeding of original 10 ml cultures to developing the chromatin assembly autoradiogram was roughly 10 days. The characterization of the mutant extract shown in Fig. 1 was recently fully described (15). The mutant strain shown actually contained mutations in three genes: *rmc1/apc5*^{CA}, *rmc2* and *rmc3/rsp5*^{CA}.

Non-radioactive *in vitro* chromatin assembly

The chromatin assembly assay used to generate Figure 1 has been described previously (11, 15). The complete experiment required approximately three days, which included running the gel overnight (for maximum separation of topoisomers) and exposure of the gel to film for an additional night. To speed up the process, we developed a non-radioactive *in vitro* chromatin assembly assay that can be completed in one day. The basic premise is identical to the radioactive assay: relaxed circular plasmid DNA is incubated with whole cell protein extract. More DNA is required (100 - 200 ng vs. 20 ng) for this reaction to allow visualization by ethidium bromide in an agarose gel. 150 ng of input plasmid gave the best ratio of intermediate to completely

supercoiled product when incubated with 200 µg of protein extract (Fig. 2A).

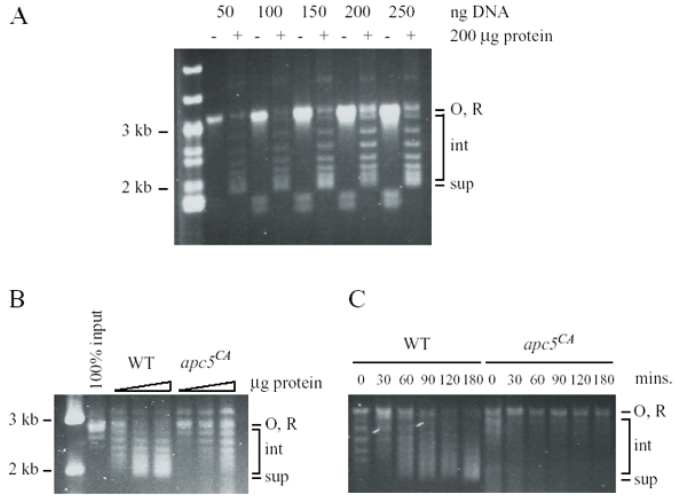

Fig. 2: Yeast whole-cell extracts support a rapid, non-radioactive *in vitro* chromatin assembly reaction. A. Increasing amounts of relaxed plasmid DNA were incubated with 200 µg of wild type (YTH1235) extract protein. The reaction proceeded for 1 hour at 30°C. The reaction products were then separated through a 1% agarose gel, stained with ethidium bromide, exposed to an ultraviolet (UV) radiation source and photographed. **B.** Increasing amounts of extract protein from wild type (YTH1235) and *apc5*^{CA} (YTH1155) cells were incubated with 200 ng of relaxed plasmid DNA. The *apc5*^{CA} extract remained defective in this modified assay. **C.** A time course was established with a reaction containing 200 ng of relaxed plasmid DNA and 200 µg of either wild type or *apc5*^{CA} extract protein (strains were as described in **Fig. 2B**). Following the reaction, the plasmid DNA was precipitated and analyzed as described above.

We also increased the amount of protein extract added to the reaction (200 - 300 µg vs. 50 - 100 µg) to compensate for the extra template DNA (Fig. 2B). In the experiment shown in Fig. 2B, 200 µg of protein was sufficient to supercoil 200 ng of input plasmid. Increasing the amount of extract past 200 ng did not increase the supercoiling efficiency. The reaction requires 2 mM ATP (data not shown), an incubation time of up to three hours to completely assemble the input plasmid (Fig. 2C) and can be conducted in a single day. Yeast strains shown to be defective when using the radioactive assay (15) remain defective when using the rapid non-radioactive assay (*apc5*^{CA} lanes in Fig. 2B and 2C). Therefore, use of the non-radioactive chromatin assembly assay when screening extracts for the purpose of identifying chromatin assembly impaired extracts greatly reduces the total time required for analysis.

In vivo chromatin assembly

To confirm that the *in vitro* chromatin assembly defects we observed reflected compromised *in vivo* chromatin metabolism, we took advantage of the supercoiling capacity of the yeast endogenous 2µ plasmid, demonstrated by Grunstein and colleagues (28, 29). To assess the supercoiling capacity of the 2µ plasmid, we grew cells in 10 ml of the required media and harvested the cells at mid log (OD_{600} 1-3). Total DNA was then isolated according to standard protocols (29; see Materials and Methods for our modifications). Initially, 10 µg of total DNA was separated through a 0.8% agarose gel containing the intercalating agent, chloroquine. The amount of 2µ plasmid recovered varies between preparations and strains. Therefore, we generally adjust the load of DNA in subsequent runs. We use 2.0 µg/ml chloroquine in the initial gels and running buffer and adjust accordingly afterwards in order to observe maximum supercoiling.

The *in vivo* chromatin assembly activity depends on histones H3 and H4. Our experiments demonstrated that 2µ plasmids isolated from cells where histones H3 and H4 were depleted have a reduced capacity to supercoil the 2µ plasmid (Fig. 3A). A strain containing deletions of the histone H3 and H4 encoding genes can be maintained by a plasmid expressing H3 and H4 from the *GAL1/10* promoter. In the presence of galactose, histones H3 and H4 are expressed. However, in the presence of glucose, the *GAL1/10* promoter is repressed, resulting in the depletion of H3 and H4 and a loss of 2µ supercoiling. This has been shown previously and serves as a control in our experiments (15, 29). Furthermore, we have previously shown that *in vitro* chromatin assembly is also compromised in these cells when grown in glucose (11).

The *in vitro* and *in vivo* assembly assays define distinct assembly activities

We next asked whether the similar histone dependence observed with the *in vitro* and *in vivo* assays reflected similar molecular mechanisms shared by the two activities. First, we determined the *in vivo* chromatin assembly profiles of two known *in vitro* chromatin assembly mutants, *apc5*^{CA} and *rsp5*^{CA}. We had already shown that the *rsp5*^{CA} mutant was indeed chromatin assembly defective *in vivo* (15), but surprisingly, the *apc5*^{CA} mutant was completely wild type in this assay, even when the 2µ plasmid was extracted from cells grown at 37°C (Fig. 3B). This was the first indication that the two assays were fundamentally different.

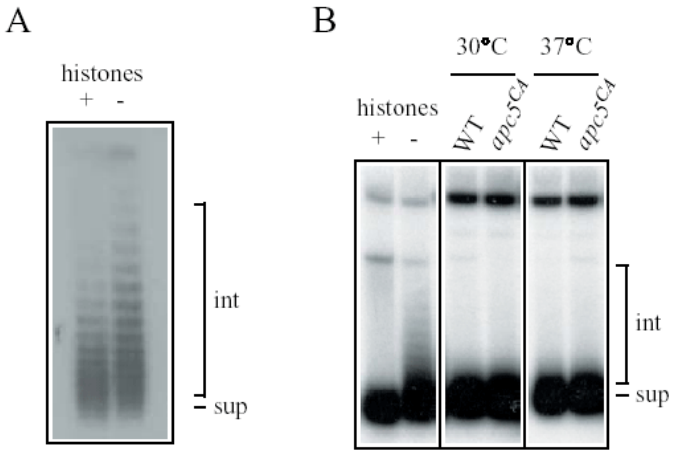

Fig. 3: An *in vivo* chromatin assembly assay assessing the supercoiling capacity of the yeast endogenous 2µ plasmid. A. The *in vivo* assay is dependent upon histones. A plasmid encoding histone H3 and H4, under the control of the *GAL1/10* promoter, was expressed in a strain harboring deletions

of genes encoding histone H3 and H4 (RMY102; a generous gift from M. Grunstein). RMY102 was either grown in galactose to express H3 and H4, or grown in galactose and then shifted to glucose, which represses histone H3 and H4 synthesis, and therefore depletes histone H3 and H4. 2µ plasmids recovered from RMY102 grown under these conditions reveals a dependence on histones (compare the accumulation of intermediate topoisomers [int] in the – histone lane to the + histone lane). The 2µ plasmid was separated through an agarose gel containing 2.0 mg chloroquine and visualized using a Southern analysis with ^{32}P labeled *REP1* PCR products (see Materials and Methods). **B.** An *apc5^{CA}* mutation, which confers *in vitro* chromatin assembly defects, has no effect on *in vivo* chromatin assembly. Isogenic wild type (YTH6) and *apc5^{CA}* (YTH1155) cells were grown at 30°C and harvested, or shifted to 37°C, the *apc5^{CA}* restrictive temperature, for an additional day of growth. 2µ plasmids were recovered and analyzed by agarose gel electrophoresis and Southern hybridization. RMY102 cells, grown in galactose (+ histones) or glucose (- histones), were included as controls. The gel and buffer contained 2.5 µg/ml chloroquine in this experiment.

With the knowledge that the two assays may indeed represent different activities, we next asked whether the two activities differed in response to cell cycle cues. The *in vitro* chromatin assembly activity is cell cycle dependent, with most activity occurring during mitosis (14, 15). To test whether the *in vivo* activity also relied on the cell cycle cues, we performed an arrest/release experiment with wild type cells. A fresh overnight culture was arrested with 300 mM hydroxyurea for 3 hours at room temperature. Since the cells used in this study expressed the wild type version of the Bar1p protease that degrades alpha factor, hydroxyurea was used, rather than alpha factor, to arrest the cells in G1. Hydroxyurea was then washed away and the cells were resuspended in fresh YPD media lacking hydroxyurea and the incubation was continued. Aliquots were removed after 60, 90 and 120 minutes for FACS analysis and 2µ plasmid isolation. The FACS data demonstrated that the wild type cells re-entered the cell cycle (Fig. 4A). However, the 2µ plasmid assembly profiles showed that there was no significant difference in supercoiling capacity between plasmids isolated from G1/S or G2/M (compare lanes 3 with lanes 4 and 5 in Fig. 4B).

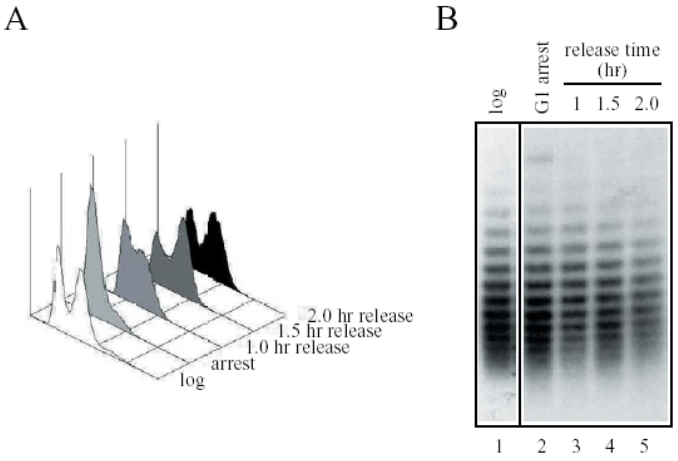

Fig. 4: The *in vivo* chromatin assembly assay does not respond to cell cycle cues, unlike the mitotic specific *in vitro* assay. A. Wild type (YTH6) yeast cells were grown to mid log (OD_{600} of 1.0-1.5) and arrested in the G1 phase of the cell cycle by growth in the presence of 300 mM hydroxyurea for 3 hours. Following this treatment, the cells were washed with fresh YPD media, added back to hydroxyurea-free YPD and allowed to re-enter the cell cycle. Aliquots were removed at the indicated time points for FACS analysis and 2µ plasmid recovery. The FACS profiles demonstrated that the cells re-entered the cell cycle

Therefore, this analysis shows that the *in vitro* and *in vivo* assays respond to different signals within cells. This is also consistent with the observation that *rsp5^{CA}*, but not *apc5^{CA}* cells, are defective for *in vivo* chromatin assembly. The APC, of which Apc5p is a subunit, functions to ensure progression through, and exit from mitosis is unimpeded (24). Rsp5p, on the other hand, has many pleiotropic effects in the cell that do not depend on the cell cycle, such as endocytosis, nutrient sensing, mitochondrial biogenesis, signal transduction and RNA PolII-dependent transcription (21-23). Therefore, Rsp5p may be required to impinge upon both the cell cycle-independent *in vivo* chromatin assembly machinery and the mitotic *in vitro* chromatin assembly machinery, in order to carry out its many functions.

CONCLUSIONS

In this report we describe protocols designed to investigate chromatin assembly in the model system, *Saccharomyces cerevisiae*. An *in vitro* chromatin assembly assay has been developed and utilized to screen yeast mutants for defects in chromatin assembly. This report describes modifications on the procedure first published in 1997 (11) that makes screening 100 extracts a manageable exercise. We also describe a rapid protocol to analyze extracts for *in vitro* activity without the use of radioactivity. Furthermore, we show the utility of an *in vivo* chromatin assembly assay, initially described by Grunstein and colleagues (28, 29), for further analysis of mutants defective for *in vitro* assembly. The results demonstrate that the two assays define separate chromatin assembly control networks that can be impinged upon by factors that can utilize one, or both activities. Thus, the tools described in this report enable factors involved in chromatin assembly throughout the cell cycle to be isolated via genetic screens and to be rigorously characterized. Indeed, we have found that mutants known to be defective for S phase coupled chromatin assembly, when in certain combinations, are compromised for *in vitro* chromatin assembly (Harkness and Arnason, unpublished data). Current efforts are aimed at determining whether the whole-cell extract preparation described here is amenable to systems other than yeast.

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